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(54) Title: THIOREDOXIN H HOMOLOGS

(54) Titre: HOMOLOGUES DE THIOREDOXINE H

(57) Abstract

This invention relates to an isolated nucleic acid fragment encoding a thioredoxin protein. The invention also relates to the construction of a chimeric gene encoding all or a portion of the thioredoxin protein, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the thioredoxin protein in a transformed host cell.

(57) Abrégé

La présente invention concerne un fragment d'acide nucléique codant une protéine thioredoxine. Cette invention concerne également l'élaboration d'un gène chimère codant la totalité ou une partie de la protéine thioredoxine, dans une orientation sens ou antisens; l'expression du gène chimère aboutissant à la production de taux modifiés de la protéine thioredoxine dans une cellule hôte transformée.

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(54) Title: THIOREDOXIN H HOMOLOGS

(57) Abstract

This invention relates to an isolated nucleic acid fragment encoding a thioredoxin protein. The invention also relates to the construction of a chimeric gene encoding all or a portion of the thioredoxin protein, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of the thioredoxin protein in a transformed host cell.

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Description

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TITLE

THIOREDOXIN H HOMOLOGS

This application claims the benefit of U.S. Provisional Application No. 60/099,501, filed September 8, 1998.

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FIELD OF THE INVENTION

This invention is in the field of plant molecular biology. More specifically, this invention pertains to nucleic acid fragments encoding thioredoxin proteins in plants and seeds.

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BACKGROUND OF THE INVENTION

10 Thioredoxin H is a cytosolic member of the thioredoxin family of proteins. These small proteins (typical mass of 12 kD) have been shown to play a central role in the activation of proteins by influencing the redox status of sulphhydryl groups on target proteins (Holmgren et al. (1985) *Annu. Rev. Biochem* 54:237-271; and Holmgren et al. (1995) *Structure* 3(3):239-243). Two other thioredoxin classes, F and M, are located in plastids and 20 have been shown to be involved in redox mediated activation/inactivation of various photosynthetic enzymes during light/dark transitions. The cytosolic (H) form of thioredoxin has been shown to be involved in disassembly of seed storage proteins during germination 25 and in the bread making process. In the former case storage proteins are held together in clusters by S-S bonds. On germination thioredoxin H reduces the S-S bonds and the 30 subunits dissociate, facilitating attack by proteases. During bread making the same processes occur. Reduction of the S-S bonds causes the protein complexes to disassemble allowing them to be distributed through out the dough during mixing. During kneading the S-H bonds become oxidized and start to reassociate in a random manner, the cross linked matrix formed by this process entraps CO₂ formed during yeast fermentation and is 35 responsible for the raising process. Addition of thioredoxin H to poor quality flours improves their quality for the production of bread.

35 Thioredoxin H has also been shown to inactivate snake and bee venom toxins and has been shown to reduce the allergenicity of cereal proteins. In the later, the process is presumably the same as described above; by reducing the S-S bonds holding the storage 40 protein clusters together they are more susceptible to denaturation and proteolysis in the gut. Thioredoxin H may also be overexpressed in transformed corn kernels and other cereal crops. The wet milling industry, which is primarily focused on starch extraction, steeps corn in liquors of sodium metabisulphite or SO₂. Although this has many secondary effects (e.g., suppression of microbial activity), the primary function is to cause a dissociation of the 45 storage proteins which leads to more efficient starch extraction. Small increases in extractable starch translate into significant increases in the profit margins for the wet millers. By overexpressing thioredoxin H in maize kernels and other cereals it may possible to 50 improve starch recoveries, reduce steep times, and reduce or eliminate the use of sulfur

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5 compounds in the steeping process. Overexpression of thioredoxin H in maize kernels and other cereals may have the added advantage of reducing the allergenicity of any transgenic protein engineered into cereal crops with high sulphydryl content.

SUMMARY OF THE INVENTION

10 5 The present invention relates to isolated polynucleotides comprising a nucleotide sequence encoding a first polypeptide of at least 100 amino acids that has at least 80% identity based on the Clustal Method of alignment when compared to a polypeptide selected from the group consisting of a *Momordica charantia* thioredoxin polypeptide of SEQ ID NO:2, a *Catakaoa speciosa* thioredoxin polypeptide of SEQ ID NO:4, a soybean thioredoxin polypeptide of SEQ ID NO:6, a soybean thioredoxin polypeptide of SEQ ID NO:8, and a *Vernonia* thioredoxin polypeptide of SEQ ID NO:10. The present invention also relates to an isolated polynucleotide comprising the complement of the nucleotide sequences described above. It is preferred that the isolated polynucleotides of the claimed invention consists of regions of the isolated polynucleotide selected from the group SEQ ID NO:1, 3, 5, 7 and 9 that codes for the polypeptide selected from the group consisting of SEQ ID NO:2, 4, 6, 8 and 10. The present invention also relates to an isolated polynucleotide comprising a nucleotide sequences of at least one of 40 (preferably 30) contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NO:1, 3, 5, 7, 9 and the complement of such nucleotide sequences.

15 10 20 15 25 30 35 40 45 50 The present invention relates to a chimeric gene comprising an isolated polynucleotide of the present invention operably linked to suitable regulatory sequences. The present invention relates to an isolated host cell comprising a chimeric gene of the present invention or an isolated polynucleotide of the present invention. The host cell may be eucaryotic, such as a yeast or a plant cell, or prokaryotic, such as a bacterial cell. The present invention also relates to a virus, preferably a baculovirus, comprising an isolated polynucleotide of the present invention or a chimeric gene of the present invention. The present invention relates to a process for producing an isolated host cell comprising a chimeric gene or isolated polynucleotide of the present invention, the process comprising either transforming or transfecting an isolated compatible host cell with a chimeric gene or isolated polynucleotide of the present invention. The present invention relates to a thioredoxin polypeptide of at least 100 amino acids comprising at least 80% homology based on the Clustal method of alignment compared to a polypeptide selected from the group consisting of SEQ ID NO:2, 4, 6, 8 and 10. The present invention relates to a method of selecting an isolated polynucleotide that affects the level of expression of a thioredoxin polypeptide in a plant cell, the method comprising the steps of: constructing an isolated polynucleotide or chimeric gene of the present invention;

5 introducing the isolated polynucleotide into a plant cell;
measuring the level of thioredoxin polypeptide in the plant cell containing the
polynucleotide; and
comparing the level of thioredoxin polypeptide in the plant cell containing the
10 5 isolated polynucleotide with the level of thioredoxin polypeptide in a plant cell that does not
contain the isolated polynucleotide.

15 The present invention relates to a method of obtaining a nucleic acid fragment
encoding a substantial portion of a thioredoxin gene, preferably a plant thioredoxin gene,
comprising the steps of: synthesizing an oligonucleotide primer comprising a nucleotide
10 10 sequence of at least one of 40 (preferably 30) contiguous nucleotides derived from a
nucleotide sequence selected from the group consisting of SEQ ID NO:1, 3, 5, 7, 9 and the
complement of such nucleotide sequences; and amplifying a nucleic acid fragment
(preferably a cDNA inserted in a cloning vector) using the oligonucleotide primer. The
20 20 amplified nucleic acid fragment preferably will encode a portion of a thioredoxin
15 polypeptide amino acid sequence.

25 The present invention also relates to a method of obtaining a nucleic acid fragment
encoding all or a substantial portion of the amino acid sequence encoding a thioredoxin
protein comprising the steps of: probing a cDNA or genomic library with an isolated
polynucleotide of the present invention; identifying a DNA clone that hybridizes with an
20 20 isolated polynucleotide of the present invention; isolating the identified DNA clone; and
sequencing the cDNA or genomic fragment that comprises the isolated DNA clone.

30 BRIEF DESCRIPTION OF THE SEQUENCE DESCRIPTIONS

The invention can be more fully understood from the following detailed description
and the accompanying Sequence Listing which form a part of this application.

35 25 Table 1 lists the polypeptides that are described herein, the designation of the cDNA
clones that comprise the nucleic acid fragments encoding polypeptides representing all or a
substantial portion of these polypeptides, and the corresponding identifier (SEQ ID NO:) as
used in the attached Sequence Listing. The sequence descriptions and Sequence Listing
attached hereto comply with the rules governing nucleotide and/or amino acid sequence
40 30 disclosures in patent applications as set forth in 37 C.F.R. §1.821-1.825.

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TABLE 1
Thioredoxin Proteins

Protein	Clone Designation	(Nucleotide)	SEQ ID NO: (Amino Acid)
Thioredoxin H	fds.pk0001.e9	1	2
Thioredoxin H	ncs.pk0010.e3	3	4
Thioredoxin H	sah1c.pk001.l17	5	6
Thioredoxin H	sf11.pk0029.e2	7	8
Thioredoxin H	vs1n.pk0012.f3	9	10

The Sequence Listing contains the one letter code for nucleotide sequence characters.

5 and the three letter codes for amino acids which is in conformity with the IUPAC-IUBMB standards described in *Nucleic Acids Res.* 13:3021-3030 (1985) and in the *Biochemical J.* 219 (No. 2):345-373 (1984) which are herein incorporated by reference. The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

10 DETAILED DESCRIPTION OF THE INVENTION

In the context of this disclosure, a number of terms shall be utilized. As used herein, a "polynucleotide" is a nucleotide sequence such as a nucleic acid fragment. A polynucleotide may be a polymer of RNA or DNA that is single- or double-stranded, that optionally contains synthetic, non-natural or altered nucleotide bases. A polynucleotide in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA, or synthetic DNA.

As used herein, "substantially similar" refers to nucleic acid fragments wherein changes in one or more nucleotide bases results in substitution of one or more amino acids, but do not affect the functional properties of the polypeptide encoded by the nucleotide

20 sequence. "Substantially similar" also refers to nucleic acid fragments wherein changes in one or more nucleotide bases does not affect the ability of the nucleic acid fragment to mediate alteration of gene expression by gene silencing through for example antisense or co-suppression technology. "Substantially similar" also refers to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more

25 nucleotides that do not substantially affect the functional properties of the resulting transcript vis-à-vis the ability to mediate gene silencing or alteration of the functional properties of the resulting protein molecule. It is therefore understood that the invention encompasses more than the specific exemplary nucleotide or amino acid sequences and includes functional equivalents thereof.

30 For example, it is well known in the art that antisense suppression and co-suppression of gene expression may be accomplished using nucleic acid fragments representing less than

5 the entire coding region of a gene, and by nucleic acid fragments that do not share 100%—
sequence identity with the gene to be suppressed. Moreover, alterations in a nucleic acid
fragment which result in the production of a chemically equivalent amino acid at a given
site, but do not effect the functional properties of the encoded polypeptide, are well known in
10 the art. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be
substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more
hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result
in substitution of one negatively charged residue for another, such as aspartic acid for
15 glutamic acid, or one positively charged residue for another, such as lysine for arginine, can
also be expected to produce a functionally equivalent product. Nucleotide changes which
result in alteration of the N-terminal and C-terminal portions of the polypeptide molecule
would also not be expected to alter the activity of the polypeptide. Each of the proposed
20 modifications is well within the routine skill in the art, as is determination of retention of
biological activity of the encoded products. Consequently, a polynucleotide comprising a
25 nucleotide sequence of at least one of 40 (preferably 30) contiguous nucleotides derived
from a nucleotide sequence selected from the group consisting of SEQ ID NO:1, 3, 5, 7, 9
and the complement of such nucleotide sequences may be used in methods of selecting an
isolated polynucleotide that affects the expression of a thioredoxin polypeptide in a plant
cell.

20 Moreover, substantially similar nucleic acid fragments may also be characterized by
their ability to hybridize. Estimates of such homology are provided by either DNA-DNA or
30 DNA-RNA hybridization under conditions of stringency as is well understood by those
skilled in the art (Hames and Higgins, Eds. (1985) Nucleic Acid Hybridisation, IRL Press,
Oxford, U.K.). Stringency conditions can be adjusted to screen for moderately similar
35 fragments, such as homologous sequences from distantly related organisms, to highly similar
fragments, such as genes that duplicate functional enzymes from closely related organisms.
Post-hybridization washes determine stringency conditions. One set of preferred conditions
uses a series of washes starting with 6X SSC, 0.5% SDS at room temperature for 15 min.
40 then repeated with 2X SSC, 0.5% SDS at 45°C for 30 min, and then repeated twice with
0.2X SSC, 0.5% SDS at 50°C for 30 min. A more preferred set of stringent conditions uses
higher temperatures in which the washes are identical to those above except for the
temperature of the final two 30 min washes in 0.2X SSC, 0.5% SDS was increased to 60°C.
45 Another preferred set of highly stringent conditions uses two final washes in 0.1X SSC,
0.1% SDS at 65°C.

35 Substantially similar nucleic acid fragments of the instant invention may also be
characterized by the percent identity of the amino acid sequences that they encode to the
amino acid sequences disclosed herein, as determined by algorithms commonly employed by
those skilled in this art. Suitable nucleic acid fragments (polynucleotides) encode amino
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5 acid sequences that are 80% identical to the amino acid sequences reported herein. Preferred
nucleic acid fragment encode amino acid sequences that are 85% identical to the amino acid
sequences reported herein. More preferred nucleic acid fragments encode amino acid
sequences that are 90% identical to the amino acid sequences reported herein. Most
10 preferred are nucleic acid fragments that encode amino acid sequences that are 95% identical
to the amino acid sequences reported herein. Sequence alignments and percent identity
calculations were performed using the Megalign program of the LASERGENE
15 bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the
sequences was performed using the Clustal method of alignment (Higgins and Sharp (1989)
10 *CABIOS* 5:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH
PENALTY=10). Default parameters for pairwise alignments using the Clustal method were
KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5.

20 A "substantial portion" of an amino acid or nucleotide sequence comprises an amino
acid or a nucleotide sequence that is sufficient to afford putative identification of the protein
25 or gene that the amino acid or nucleotide sequence comprises. Amino acid and nucleotide
sequences can be evaluated either manually by one skilled in the art, or by using computer-
based sequence comparison and identification tools that employ algorithms such as BLAST
(Basic Local Alignment Search Tool; Altschul et al. (1993) *J. Mol. Biol.* 215:403-410; see
also www.ncbi.nlm.nih.gov/BLAST/). In general, a sequence of ten or more contiguous
30 amino acids or thirty or more contiguous nucleotides is necessary in order to putatively
identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene.
Moreover, with respect to nucleotide sequences, gene-specific oligonucleotide probes
comprising 30 or more contiguous nucleotides may be used in sequence-dependent methods
35 of gene identification (e.g., Southern hybridization) and isolation (e.g., *in situ* hybridization
of bacterial colonies or bacteriophage plaques). In addition, short oligonucleotides of 12 or
more nucleotides may be used as amplification primers in PCR in order to obtain a particular
nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of a
40 nucleotide sequence comprises a nucleotide sequence that will afford specific identification
and/or isolation of a nucleic acid fragment comprising the sequence. The instant
specification teaches amino acid and nucleotide sequences encoding polypeptides that
comprise one or more particular plant proteins. The skilled artisan, having the benefit of the
45 sequences as reported herein, may now use all or a substantial portion of the disclosed
sequences for purposes known to those skilled in this art. Accordingly, the instant invention
comprises the complete sequences as reported in the accompanying Sequence Listing, as
well as substantial portions of those sequences as defined above.

50 "Codon degeneracy" refers to divergence in the genetic code permitting variation of
the nucleotide sequence without effecting the amino acid sequence of an encoded
polypeptide. Accordingly, the instant invention relates to any nucleic acid fragment

5 comprising a nucleotide sequence that encodes all or a substantial portion of the amino acid sequences set forth herein. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid.

10 Therefore, when synthesizing a nucleic acid fragment for improved expression in a host cell, 5 it is desirable to design the nucleic acid fragment such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

15 "Synthetic nucleic acid fragments" can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art. These building blocks are ligated and annealed to form larger nucleic acid fragments which

20 10 may then be enzymatically assembled to construct the entire desired nucleic acid fragment. "Chemically synthesized", as related to nucleic acid fragment, means that the component nucleotides were assembled *in vitro*. Manual chemical synthesis of nucleic acid fragments may be accomplished using well established procedures, or automated chemical synthesis 25 can be performed using one of a number of commercially available machines. Accordingly,

30 15 the nucleic acid fragments can be tailored for optimal gene expression based on optimization of nucleotide sequence to reflect the codon bias of the host cell. The skilled artisan appreciates the likelihood of successful gene expression if codon usage is biased towards 25 those codons favored by the host. Determination of preferred codons can be based on a survey of genes derived from the host cell where sequence information is available.

35 20 "Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature.

40 25 Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature.

45 30 "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene 50 that has been introduced into the genome by a transformation procedure.

55 35 "Coding sequence" refers to a nucleotide sequence that codes for a specific amino acid sequence. "Regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences

5 “Promoter” refers to a nucleotide sequence capable of controlling the expression of a
coding sequence or functional RNA. In general, a coding sequence is located 3' to a
promoter sequence. The promoter sequence consists of proximal and more distal upstream
elements, the latter elements often referred to as enhancers. Accordingly, an “enhancer” is a
10 5 nucleotide sequence which can stimulate promoter activity and may be an innate element of
the promoter or a heterologous element inserted to enhance the level or tissue-specificity of
a promoter. Promoters may be derived in their entirety from a native gene, or be composed
of different elements derived from different promoters found in nature, or even comprise
synthetic nucleotide segments. It is understood by those skilled in the art that different
15 10 promoters may direct the expression of a gene in different tissues or cell types, or at
different stages of development, or in response to different environmental conditions.
Promoters which cause a nucleic acid fragment to be expressed in most cell types at most
20 20 times are commonly referred to as “constitutive promoters”. New promoters of various
types useful in plant cells are constantly being discovered; numerous examples may be
25 15 found in the compilation by Okamuro and Goldberg (1989) *Biochemistry of Plants* 15:1-82.
It is further recognized that since in most cases the exact boundaries of regulatory sequences
have not been completely defined, nucleic acid fragments of different lengths may have
identical promoter activity.

25 20 The “translation leader sequence” refers to a nucleotide sequence located between the
promoter sequence of a gene and the coding sequence. The translation leader sequence is
30 30 present in the fully processed mRNA upstream of the translation start sequence. The
translation leader sequence may affect processing of the primary transcript to mRNA,
mRNA stability or translation efficiency. Examples of translation leader sequences have
been described (Turner and Foster (1995) *Mol. Biotechnol.* 3:225-236).

35 25 The “3' non-coding sequences” refer to nucleotide sequences located downstream of a
coding sequence and include polyadenylation recognition sequences and other sequences
30 35 encoding regulatory signals capable of affecting mRNA processing or gene expression. The
polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid
tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is
40 40 exemplified by Ingelbrecht et al. (1989) *Plant Cell* 1:671-680.

45 45 “RNA transcript” refers to the product resulting from RNA polymerase-catalyzed
transcription of a DNA sequence. When the RNA transcript is a perfect complementary
copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA
sequence derived from posttranscriptional processing of the primary transcript and is
50 45 referred to as the mature RNA. “Messenger RNA (mRNA)” refers to the RNA that is
without introns and that can be translated into polypeptide by the cell. “cDNA” refers to a
double-stranded DNA that is complementary to and derived from mRNA. “Sense” RNA
refers to an RNA transcript that includes the mRNA and so can be translated into a

5 polypeptide by the cell. "Antisense RNA" refers to an RNA transcript that is
complementary to all or part of a target primary transcript or mRNA and that blocks the
expression of a target gene (see U.S. Patent No. 5,107,065, incorporated herein by
reference). The complementarity of an antisense RNA may be with any part of the specific
10 5 nucleotide sequence, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or
the coding sequence. "Functional RNA" refers to sense RNA, antisense RNA, ribozyme
RNA, or other RNA that may not be translated but yet has an effect on cellular processes.

15 The term "operably linked" refers to the association of two or more nucleic acid
fragments on a single nucleic acid fragment so that the function of one is affected by the
10 other. For example, a promoter is operably linked with a coding sequence when it is capable
of affecting the expression of that coding sequence (i.e., that the coding sequence is under
the transcriptional control of the promoter). Coding sequences can be operably linked to
regulatory sequences in sense or antisense orientation.

20 The term "expression", as used herein, refers to the transcription and stable
25 accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of
the invention. Expression may also refer to translation of mRNA into a polypeptide.
"Antisense inhibition" refers to the production of antisense RNA transcripts capable of
suppressing the expression of the target protein. "Overexpression" refers to the production
25 of a gene product in transgenic organisms that exceeds levels of production in normal or
non-transformed organisms. "Co-suppression" refers to the production of sense RNA
transcripts capable of suppressing the expression of identical or substantially similar foreign
30 or endogenous genes (U.S. Patent No. 5,231,020, incorporated herein by reference).

35 "Altered levels" refers to the production of gene product(s) in transgenic organisms in
amounts or proportions that differ from that of normal or non-transformed organisms.

40 "Mature" protein refers to a post-translationally processed polypeptide; i.e., one from
which any pre- or propeptides present in the primary translation product have been removed.
"Precursor" protein refers to the primary product of translation of mRNA; i.e., with pre- and
45 propeptides still present. Pre- and propeptides may be but are not limited to intracellular
localization signals.

40 A "chloroplast transit peptide" is an amino acid sequence which is translated in
conjunction with a protein and directs the protein to the chloroplast or other plastid types
present in the cell in which the protein is made. "Chloroplast transit sequence" refers to a
nucleotide sequence that encodes a chloroplast transit peptide. A "signal peptide" is an
amino acid sequence which is translated in conjunction with a protein and directs the protein
45 to the secretory system (Chrispeels (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol.* 42:21-53).
If the protein is to be directed to a vacuole, a vacuolar targeting signal (*supra*) can further be
added, or if to the endoplasmic reticulum, an endoplasmic reticulum retention signal (*supra*)
may be added. If the protein is to be directed to the nucleus, any signal peptide present

5 should be removed and instead a nuclear localization signal included (Raikhel (1992) *Plant Phys.* 100:1627-1632).

10 "Transformation" refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the
5 transformed nucleic acid fragments are referred to as "transgenic" organisms. Examples of methods of plant transformation include *Agrobacterium*-mediated transformation (De Blaere et al. (1987) *Meth. Enzymol.* 143:277) and particle-accelerated or "gene gun" transformation technology (Klein et al. (1987) *Nature (London)* 327:70-73; U.S. Patent No. 4,945,050, incorporated herein by reference).

15 10 Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook et al. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1989 (hereinafter "Maniatis").

20 15 Nucleic acid fragments encoding at least a portion of several thioredoxin proteins have been isolated and identified by comparison of random plant cDNA sequences to public databases containing nucleotide and protein sequences using the BLAST algorithms well known to those skilled in the art. The nucleic acid fragments of the instant invention may be used to isolate cDNAs and genes encoding homologous proteins from the same or other plant species. Isolation of homologous genes using sequence-dependent protocols is well
25 20 known in the art. Examples of sequence-dependent protocols include, but are not limited to, methods of nucleic acid hybridization, and methods of DNA and RNA amplification as exemplified by various uses of nucleic acid amplification technologies (e.g., polymerase chain reaction, ligase chain reaction).

30 25 For example, genes encoding other thioredoxin H proteins, either as cDNAs or genomic DNAs, could be isolated directly by using all or a portion of the instant nucleic acid fragments as DNA hybridization probes to screen libraries from any desired plant employing methodology well known to those skilled in the art. Specific oligonucleotide probes based upon the instant nucleic acid sequences can be designed and synthesized by methods known in the art (Maniatis). Moreover, the entire sequences can be used directly to synthesize
35 30 DNA probes by methods known to the skilled artisan such as random primer DNA labeling, nick translation, or end-labeling techniques, or RNA probes using available *in vitro* transcription systems. In addition, specific primers can be designed and used to amplify a part or all of the instant sequences. The resulting amplification products can be labeled directly during amplification reactions or labeled after amplification reactions, and used as
40 35 probes to isolate full length cDNA or genomic fragments under conditions of appropriate stringency.

45 50 In addition, two short segments of the instant nucleic acid fragments may be used in polymerase chain reaction protocols to amplify longer nucleic acid fragments encoding

5 homologous genes from DNA or RNA. The polymerase chain reaction may also be
performed on a library of cloned nucleic acid fragments wherin the sequence of one primer
is derived from the instant nucleic acid fragments, and the sequence of the other primer takes
advantage of the presence of the polyadenylic acid tracts to the 3' end of the mRNA

10 5 precursor encoding plant genes. Alternatively, the second primer sequence may be based
upon sequences derived from the cloning vector. For example, the skilled artisan can follow
the RACE protocol (Frohman et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:8998-9002) to
generate cDNAs by using PCR to amplify copies of the region between a single point in the
transcript and the 3' or 5' end. Primers oriented in the 3' and 5' directions can be designed

15 10 from the instant sequences. Using commercially available 3' RACE or 5' RACE systems
(BRL), specific 3' or 5' cDNA fragments can be isolated (Ohara et al. (1989) *Proc. Natl.
Acad. Sci. USA* 86:5673-5677; Loh et al. (1989) *Science* 243:217-220). Products generated
by the 3' and 5' RACE procedures can be combined to generate full-length cDNAs (Frohman
20 and Martin (1989) *Techniques* 1:165). Consequently, a polynucleotide comprising a

25 15 nucleotide sequence of at least one of 40 (preferably 30) contiguous nucleotides derived
from a nucleotide sequence selected from the group consisting of SEQ ID NO:1, 3, 5, 7, 9
and the complement of such nucleotide sequences may be used in such methods to obtain a
nucleic acid fragment encoding a substantial portion of an amino acid sequence of a
thioredoxin polypeptide.

30 20 Availability of the instant nucleotide and deduced amino acid sequences facilitates
immunological screening of cDNA expression libraries. Synthetic peptides representing
portions of the instant amino acid sequences may be synthesized. These peptides can be
used to immunize animals to produce polyclonal or monoclonal antibodies with specificity
35 25 for peptides or proteins comprising the amino acid sequences. These antibodies can be then
be used to screen cDNA expression libraries to isolate full-length cDNA clones of interest
(Lerner (1984) *Adv. Immunol.* 36:1-34; Maniatis).

35 30 The nucleic acid fragments of the instant invention may be used to create transgenic
plants in which the disclosed polypeptides are present at higher or lower levels than normal
or in cell types or developmental stages in which they are not normally found. This would
40 35 have the effect of altering the level of thioredoxin activity in those cells.

45 40 Overexpression of the proteins of the instant invention may be accomplished by first
constructing a chimeric gene in which the coding region is operably linked to a promoter
capable of directing expression of a gene in the desired tissues at the desired stage of
development. For reasons of convenience, the chimeric gene may comprise promoter
45 45 sequences and translation leader sequences derived from the same genes. 3' Non-coding
sequences encoding transcription termination signals may also be provided. The instant
chimeric gene may also comprise one or more introns in order to facilitate gene expression.

5 Plasmid vectors comprising the instant chimeric gene can then be constructed. The choice of plasmid vector is dependent upon the method that will be used to transform host plants. The skilled artisan is well aware of the genetic elements that must be present on the plasmid vector in order to successfully transform, select and propagate host cells containing
10 the chimeric gene. The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression (Jones et al. (1985) *EMBO J.* 4:2411-2418; De Almeida et al. (1989) *Mol. Gen. Genetics* 218:78-86), and thus that multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of
15 DNA, Northern analysis of mRNA expression, Western analysis of protein expression, or phenotypic analysis.

For some applications it may be useful to direct the instant polypeptides to different cellular compartments, or to facilitate its secretion from the cell. It is thus envisioned that
20 the chimeric gene described above may be further supplemented by altering the coding sequence to encode the instant polypeptides with appropriate intracellular targeting
25 sequences such as transit sequences (Keegstra (1989) *Cell* 56:247-253), signal sequences or sequences encoding endoplasmic reticulum localization (Chrispeels (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol.* 42:21-53), or nuclear localization signals (Raikhel (1992) *Plant Phys.* 100:1627-1632) added and/or with targeting sequences that are already present
30 removed. While the references cited give examples of each of these, the list is not exhaustive and more targeting signals of utility may be discovered in the future.

It may also be desirable to reduce or eliminate expression of genes encoding the instant polypeptides in plants for some applications. In order to accomplish this, a chimeric gene designed for co-suppression of the instant polypeptide can be constructed by linking a
35 gene or gene fragment encoding that polypeptide to plant promoter sequences. Alternatively, a chimeric gene designed to express antisense RNA for all or part of the instant nucleic acid fragment can be constructed by linking the gene or gene fragment in reverse orientation to plant promoter sequences. Either the co-suppression or antisense
40 chimeric genes could be introduced into plants via transformation wherein expression of the corresponding endogenous genes are reduced or eliminated.

45 Molecular genetic solutions to the generation of plants with altered gene expression have a decided advantage over more traditional plant breeding approaches. Changes in plant phenotypes can be produced by specifically inhibiting expression of one or more genes by antisense inhibition or cosuppression (U.S. Patent Nos. 5,190,931, 5,107,065 and
50 5,283,323). An antisense or cosuppression construct would act as a dominant negative regulator of gene activity. While conventional mutations can yield negative regulation of gene activity these effects are most likely recessive. The dominant negative regulation available with a transgenic approach may be advantageous from a breeding perspective. In

5 addition, the ability to restrict the expression of specific phenotype to the reproductive tissues of the plant by the use of tissue specific promoters may confer agronomic advantages relative to conventional mutations which may have an effect in all tissues in which a mutant gene is ordinarily expressed.

10 5 The person skilled in the art will know that special considerations are associated with the use of antisense or cosuppression technologies in order to reduce expression of particular genes. For example, the proper level of expression of sense or antisense genes may require the use of different chimeric genes utilizing different regulatory elements known to the skilled artisan. Once transgenic plants are obtained by one of the methods described above,

15 10 it will be necessary to screen individual transgenics for those that most effectively display the desired phenotype. Accordingly, the skilled artisan will develop methods for screening large numbers of transformants. The nature of these screens will generally be chosen on practical grounds, and is not an inherent part of the invention. For example, one can screen by looking for changes in gene expression by using antibodies specific for the protein

20 15 encoded by the gene being suppressed, or one could establish assays that specifically measure enzyme activity. A preferred method will be one which allows large numbers of samples to be processed rapidly, since it will be expected that a large number of transformants will be negative for the desired phenotype.

25 The instant polypeptides (or portions thereof) may be produced in heterologous host 20 cells, particularly in the cells of microbial hosts, and can be used to prepare antibodies to these proteins by methods well known to those skilled in the art. The antibodies are useful 30 for detecting the polypeptides of the instant invention *in situ* in cells or *in vitro* in cell extracts. Preferred heterologous host cells for production of the instant polypeptides are microbial hosts. Microbial expression systems and expression vectors containing regulatory 35 sequences that direct high level expression of foreign proteins are well known to those skilled in the art. Any of these could be used to construct a chimeric gene for production of the instant polypeptides. This chimeric gene could then be introduced into appropriate microorganisms via transformation to provide high level expression of the encoded thioredoxin protein. An example of a vector for high level expression of the instant 40 polypeptides in a bacterial host is provided (Example 6).

45 All or a substantial portion of the nucleic acid fragments of the instant invention may also be used as probes for genetically and physically mapping the genes that they are a part of, and as markers for traits linked to those genes. Such information may be useful in plant breeding in order to develop lines with desired phenotypes. For example, the instant nucleic acid fragments may be used as restriction fragment length polymorphism (RFLP) markers. 50 Southern blots (Maniatis) of restriction-digested plant genomic DNA may be probed with the nucleic acid fragments of the instant invention. The resulting banding patterns may then be subjected to genetic analyses using computer programs such as MapMaker (Lander et al.

5 (1987) *Genomics* 1:174-181) in order to construct a genetic map. In addition, the nucleic acid fragments of the instant invention may be used to probe Southern blots containing restriction endonuclease-treated genomic DNAs of a set of individuals representing parent and progeny of a defined genetic cross. Segregation of the DNA polymorphisms is noted
10 5 and used to calculate the position of the instant nucleic acid sequence in the genetic map previously obtained using this population (Botstein et al. (1980) *Am. J. Hum. Genet.* 32:314-331).

15 The production and use of plant gene-derived probes for use in genetic mapping is described in Bernatzky and Tanksley (1986) *Plant Mol. Biol. Reporter* 4:37-41. Numerous 10 publications describe genetic mapping of specific cDNA clones using the methodology outlined above or variations thereof. For example, F2 intercross populations, backcross populations, randomly mated populations, near isogenic lines, and other sets of individuals may be used for mapping. Such methodologies are well known to those skilled in the art.

20 Nucleic acid probes derived from the instant nucleic acid sequences may also be used 15 for physical mapping (i.e., placement of sequences on physical maps; see Hoheisel et al. In: *Nonmammalian Genomic Analysis: A Practical Guide*, Academic press 1996, pp. 319-346, and references cited therein).

25 In another embodiment, nucleic acid probes derived from the instant nucleic acid sequences may be used in direct fluorescence *in situ* hybridization (FISH) mapping (Trask 20 (1991) *Trends Genet.* 7:149-154). Although current methods of FISH mapping favor use of 30 large clones (several to several hundred KB; see Laan et al. (1995) *Genome Res.* 5:13-20), improvements in sensitivity may allow performance of FISH mapping using shorter probes.

35 A variety of nucleic acid amplification-based methods of genetic and physical 25 mapping may be carried out using the instant nucleic acid sequences. Examples include allele-specific amplification (Kazazian (1989) *J. Lab. Clin. Med.* 11:95-96), polymorphism of PCR-amplified fragments (CAPS; Sheffield et al. (1993) *Genomics* 16:325-332), allele-specific ligation (Landegren et al. (1988) *Science* 241:1077-1080), nucleotide extension reactions (Sokolov (1990) *Nucleic Acid Res.* 18:3671), Radiation Hybrid Mapping (Walter et al. (1997) *Nat. Genet.* 7:22-28) and Happy Mapping (Dear and Cook (1989) *Nucleic Acid Res.* 17:6795-6807). For these methods, the sequence of a nucleic acid fragment is used to 40 design and produce primer pairs for use in the amplification reaction or in primer extension reactions. The design of such primers is well known to those skilled in the art. In methods employing PCR-based genetic mapping, it may be necessary to identify DNA sequence differences between the parents of the mapping cross in the region corresponding to the 45 instant nucleic acid sequence. This, however, is generally not necessary for mapping methods.

50 Loss of function mutant phenotypes may be identified for the instant cDNA clones either by targeted gene disruption protocols or by identifying specific mutants for these

5 genes contained in a maize population carrying mutations in all possible genes (Ballinger and Benzer (1989) *Proc. Natl. Acad. Sci USA* 86:9402-9406; Koes et al. (1995) *Proc. Natl. Acad. Sci USA* 92:8149-8153; Bensen et al. (1995) *Plant Cell* 7:75-84). The latter approach may be accomplished in two ways. First, short segments of the instant nucleic acid

10 5 fragments may be used in polymerase chain reaction protocols in conjunction with a mutation tag sequence primer on DNAs prepared from a population of plants in which Mutator transposons or some other mutation-causing DNA element has been introduced (see Bensen, *supra*). The amplification of a specific DNA fragment with these primers indicates the insertion of the mutation tag element in or near the plant gene encoding the instant

15 10 polypeptides. Alternatively, the instant nucleic acid fragment may be used as a hybridization probe against PCR amplification products generated from the mutation population using the mutation tag sequence primer in conjunction with an arbitrary genomic site primer, such as that for a restriction enzyme site-anchored synthetic adaptor. With either method, a plant containing a mutation in the endogenous gene encoding the instant

20 15 polypeptides can be identified and obtained. This mutant plant can then be used to determine or confirm the natural function of the instant polypeptides disclosed herein.

EXAMPLES

25 The present invention is further defined in the following Examples, in which all parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

EXAMPLE 1

Composition of cDNA Libraries; Isolation and Sequencing of cDNA Clones

30 35 cDNA libraries representing mRNAs from various barley, *Catalpa*, pear, soybean and *Vernonia* tissues were prepared. The characteristics of the libraries are described below.

40 30 TABLE 2
cDNA Libraries from Barley, Catalpa, Pear, Soybean and Vernonia

Library	Tissue	Clone
fds	<i>Momordica charantia</i> developing seed	fds.pk0001.e9
ncs	<i>Catalpa speciosa</i> developing Seed	ncs.pk0010.e3
sah1c	Soybean sprayed with Authority herbicide.	sah1c.pk001.l17
sfl1	Soybean immature flower	sfl1.pk0029.c2
vs1n	<i>Vernonia</i> Seed*	vs1n.pk0012.f3

45 50 *This library was normalized essentially as described in U.S. Patent No. 5,482,845, incorporated herein by reference.

5 cDNA libraries may be prepared by any one of many methods available. For
example, the cDNAs may be introduced into plasmid vectors by first preparing the cDNA
libraries in Uni-ZAP™ XR vectors according to the manufacturer's protocol (Stratagene
10 Cloning Systems, La Jolla, CA). The Uni-ZAP™ XR libraries are converted into plasmid
libraries according to the protocol provided by Stratagene. Upon conversion, cDNA inserts
will be contained in the plasmid vector pBluescript. In addition, the cDNAs may be
introduced directly into pre-cut Bluescript II SK(+) vectors (Stratagene) using T4 DNA
ligase (New England Biolabs), followed by transfection into DH10B cells according to the
15 manufacturer's protocol (GIBCO BRL Products). Once the cDNA inserts are in plasmid
vectors, plasmid DNAs are prepared from randomly picked bacterial colonies containing
recombinant pBluescript plasmids, or the insert cDNA sequences are amplified via
polymerase chain reaction using primers specific for vector sequences flanking the inserted
20 cDNA sequences. Amplified insert DNAs or plasmid DNAs are sequenced in dye-primer
sequencing reactions to generate partial cDNA sequences (expressed sequence tags or
"ESTs"; see Adams et al., (1991) *Science* 252:1651-1656). The resulting ESTs are analyzed
using a Perkin Elmer Model 377 fluorescent sequencer.

25 EXAMPLE 2
Identification of cDNA Clones

20 cDNA clones encoding thioredoxin proteins were identified by conducting BLAST
(Basic Local Alignment Search Tool; Altschul et al. (1993) *J. Mol. Biol.* 215:403-410; see
30 also www.ncbi.nlm.nih.gov/BLAST/) searches for similarity to sequences contained in the
BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences
derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major
35 release of the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). The
cDNA sequences obtained in Example 1 were analyzed for similarity to all publicly
available DNA sequences contained in the "nr" database using the BLASTN algorithm
provided by the National Center for Biotechnology Information (NCBI). The DNA
40 sequences were translated in all reading frames and compared for similarity to all publicly
available protein sequences contained in the "nr" database using the BLASTX algorithm
(Gish and States (1993) *Nat. Genet.* 3:266-272) provided by the NCBI. For convenience,
the P-value (probability) of observing a match of a cDNA sequence to a sequence contained
in the searched databases merely by chance as calculated by BLAST are reported herein as
45 "pLog" values, which represent the negative of the logarithm of the reported P-value.
35 Accordingly, the greater the pLog value, the greater the likelihood that the cDNA sequence
and the BLAST "hit" represent homologous proteins.

EXAMPLE 3Characterization of cDNA Clones Encoding Thioredoxin H

The BLASTX search using the EST sequences from clones listed in Table 3 revealed similarity of the polypeptides encoded by the cDNAs to thioredoxin H from *Arabidopsis thaliana* (NCBI Identifier No. gi 267122), *Nicotiana tabacum* (NCBI Identifier No. gi 267124) and *Ricinus communis* (NCBI Identifier No. gi 1255954). Shown in Table 3 are the BLAST results for individual ESTs ("EST"), the sequences of the entire cDNA inserts comprising the indicated cDNA clones ("FIS"), or contigs assembled from two or more ESTs ("Contig"):

10

TABLE 3

BLAST Results for Sequences Encoding Polypeptides Homologous to *Arabidopsis thaliana*, *Nicotiana tabacum* and *Ricinus communis* Thioredoxin H

Clone	Status	BLAST pLog Score
fds.pk0001.e9	FIS	35.50 (gi 267122)
ncs.pk0010.e3	FIS	48.40 (gi 267124)
sah1c.pk001.l17	FIS	49.00 (gi 1255954)
sfl1.pk0029.e2	FIS	41.00 (gi 1255954)
vs1n.pk0012.f3	FIS	41.70 (gi 267124)

15

The data in Table 4 represents a calculation of the percent identity of the amino acid sequences set forth in SEQ ID NOS:2, 4, 6, 8 and 10 and the *Arabidopsis thaliana*, *Nicotiana tabacum* and *Ricinus communis* sequences (SEQ ID NOS:11, 12 and 13 respectively). The percent identity between the amino acid sequences set forth in SEQ ID NOS:2, 4, 6, 8 and 10 ranged from 49% to 80%.

20

TABLE 4

Percent Identity of Amino Acid Sequences Deduced From the Nucleotide Sequences of cDNA Clones Encoding Polypeptides Homologous to *Arabidopsis thaliana*, *Nicotiana tabacum* and *Ricinus communis* Thioredoxin H

SEQ ID NO.	Percent Identity to
2	62% (gi 267122)
4	75% (gi 267124)
6	75% (gi 1255954)
8	65% (gi 1255954)
10	69% (gi 267124)

25

Sequence alignments and percent identity calculations were performed using the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal

50

5 method of alignment (Higgins and Sharp (1989) *CABIOS* 5:151-153) with the default
parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for
pairwise alignments using the Clustal method were KTUPLE 1, GAP PENALTY=3,
WINDOW=5 and DIAGONALS SAVED=5. Sequence alignments and BLAST scores and
10 probabilities indicate that the nucleic acid fragments comprising the instant cDNA clones
encode a substantial portion of a thioredoxin H. These sequences represent the first *Catalpa*,
pear, soybean and *Veronica* sequences encoding thioredoxin H.

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EXAMPLE 4

Expression of Chimeric Genes in Monocot Cells

10 A chimeric gene comprising a cDNA encoding the instant polypeptides in sense
orientation with respect to the maize 27 kD zein promoter that is located 5' to the cDNA
fragment, and the 10 kD zein 3' end that is located 3' to the cDNA fragment, can be
constructed. The cDNA fragment of this gene may be generated by polymerase chain
reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites
15 (NcoI or SmaI) can be incorporated into the oligonucleotides to provide proper orientation
of the DNA fragment when inserted into the digested vector pML103 as described below.
Amplification is then performed in a standard PCR. The amplified DNA is then digested
20 with restriction enzymes NcoI and SmaI and fractionated on an agarose gel. The appropriate
band can be isolated from the gel and combined with a 4.9 kb NcoI-SmaI fragment of the
plasmid pML103. Plasmid pML103 has been deposited under the terms of the Budapest
25 Treaty at ATCC (American Type Culture Collection, 10801 University Blvd., Manassas,
VA 20110-2209), and bears accession number ATCC 97366. The DNA segment from
pML103 contains a 1.05 kb Sall-NcoI promoter fragment of the maize 27 kD zein gene and
a 0.96 kb SmaI-Sall fragment from the 3' end of the maize 10 kD zein gene in the vector
30 pGem9Zf(+) (Promega). Vector and insert DNA can be ligated at 15°C overnight,
essentially as described (Maniatis). The ligated DNA may then be used to transform *E. coli*
35 XL1-Blue (Epicurian Coli XL-1 Blue™; Stratagene). Bacterial transformants can be
screened by restriction enzyme digestion of plasmid DNA and limited nucleotide sequence
analysis using the dideoxy chain termination method (Sequenase™ DNA Sequencing Kit;
40 U.S. Biochemical). The resulting plasmid construct would comprise a chimeric gene
encoding, in the 5' to 3' direction, the maize 27 kD zein promoter, a cDNA fragment
encoding the instant polypeptides, and the 10 kD zein 3' region.

45 The chimeric gene described above can then be introduced into corn cells by the
following procedure. Immature corn embryos can be dissected from developing caryopses
45 35 derived from crosses of the inbred corn lines H99 and LH132. The embryos are isolated 10
to 11 days after pollination when they are 1.0 to 1.5 mm long. The embryos are then placed
with the axis-side facing down and in contact with agarose-solidified N6 medium (Chu et al.
(1975) *Sci. Sin. Peking* 18:659-668). The embryos are kept in the dark at 27°C. Friable

5 embryogenic callus consisting of undifferentiated masses of cells with somatic
proembryoids and embryoids borne on suspensor structures proliferates from the scutellum
of these immature embryos. The embryogenic callus isolated from the primary explant can
be cultured on N6 medium and sub-cultured on this medium every 2 to 3 weeks.

10 5 The plasmid, p35S/Ac (obtained from Dr. Peter Eckes, Hoechst Ag, Frankfurt,
Germany) may be used in transformation experiments in order to provide for a selectable
marker. This plasmid contains the *Pat* gene (see European Patent Publication 0 242 236)
which encodes phosphinothricin acetyl transferase (PAT). The enzyme PAT confers
resistance to herbicidal glutamine synthetase inhibitors such as phosphinothricin. The *pat*
15 10 gene in p35S/Ac is under the control of the 35S promoter from Cauliflower Mosaic Virus
(Odell et al. (1985) *Nature* 313:810-812) and the 3' region of the nopaline synthase gene
from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*.

20 20 The particle bombardment method (Klein et al. (1987) *Nature* 327:70-73) may be used
to transfer genes to the callus culture cells. According to this method, gold particles (1 μ m
25 15 in diameter) are coated with DNA using the following technique. Ten μ g of plasmid DNAs
are added to 50 μ L of a suspension of gold particles (60 mg per mL). Calcium chloride
(50 μ L of a 2.5 M solution) and spermidine free base (20 μ L of a 1.0 M solution) are added
to the particles. The suspension is vortexed during the addition of these solutions. After
10 minutes, the tubes are briefly centrifuged (5 sec at 15,000 rpm) and the supernatant
30 20 removed. The particles are resuspended in 200 μ L of absolute ethanol, centrifuged again
and the supernatant removed. The ethanol rinse is performed again and the particles
resuspended in a final volume of 30 μ L of ethanol. An aliquot (5 μ L) of the DNA-coated
35 25 gold particles can be placed in the center of a KaptonTM flying disc (Bio-Rad Labs). The
particles are then accelerated into the corn tissue with a BiolisticTM PDS-1000/He (Bio-Rad
Instruments, Hercules CA), using a helium pressure of 1000 psi, a gap distance of 0.5 cm
and a flying distance of 1.0 cm.

40 35 For bombardment, the embryogenic tissue is placed on filter paper over agarose-
solidified N6 medium. The tissue is arranged as a thin lawn and covered a circular area of
about 5 cm in diameter. The petri dish containing the tissue can be placed in the chamber of
30 40 the PDS-1000/He approximately 8 cm from the stopping screen. The air in the chamber is
then evacuated to a vacuum of 28 inches of Hg. The macrocarrier is accelerated with a
helium shock wave using a rupture membrane that bursts when the He pressure in the shock
tube reaches 1000 psi.

45 45 Seven days after bombardment the tissue can be transferred to N6 medium that
35 contains glufosinate (2 mg per liter) and lacks casein or proline. The tissue continues to
grow slowly on this medium. After an additional 2 weeks the tissue can be transferred to
fresh N6 medium containing glufosinate. After 6 weeks, areas of about 1 cm in diameter
50 50 of actively growing callus can be identified on some of the plates containing the glufosinate-

supplemented medium. These calli may continue to grow when sub-cultured on the selective medium.

Plants can be regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks the tissue can be transferred to regeneration medium (Fromm et al. (1990) *Bio/Technology* 8:833-839).

EXAMPLE 5

Expression of Chimeric Genes in Dicot Cells

A seed-specific expression cassette composed of the promoter and transcription

terminator from the gene encoding the β subunit of the seed storage protein phaseolin from the bean *Phaseolus vulgaris* (Doyle et al. (1986) *J. Biol. Chem.* 261:9228-9238) can be used for expression of the instant polypeptides in transformed soybean. The phaseolin cassette includes about 500 nucleotides upstream (5') from the translation initiation codon and about 1650 nucleotides downstream (3') from the translation stop codon of phaseolin. Between the 5' and 3' regions are the unique restriction endonuclease sites Nco I (which includes the ATG translation initiation codon), SmaI, KpnI and XbaI. The entire cassette is flanked by Hind III sites.

The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the expression vector. Amplification is then performed as described above, and the isolated fragment is inserted into a pUC18 vector carrying the seed expression cassette.

Soybean embryos may then be transformed with the expression vector comprising sequences encoding the instant polypeptides. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface sterilized, immature seeds of the soybean cultivar A2872, can be cultured in the light or dark at 26°C on an appropriate agar medium for 6-10 weeks. Somatic embryos which produce secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos which multiplied as early, globular staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures can be maintained in 35 mL liquid media on a rotary shaker, 150 rpm, at 26°C with fluorescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 mL of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein et al. (1987) *Nature* (London) 327:70-73, U.S. Patent

5 No. 4,945,050). A DuPont Biolistic™ PDS1000/HE instrument (helium retrofit) can be used
for these transformations.

10 A selectable marker gene which can be used to facilitate soybean transformation is a
chimeric gene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell et al.
15 5 (1985) *Nature* 313:810-812), the hygromycin phosphotransferase gene from plasmid pJR225
(from *E. coli*; Gritz et al. (1983) *Gene* 25:179-188) and the 3' region of the nopaline synthase
20 gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The seed expression
cassette comprising the phaseolin 5' region, the fragment encoding the instant polypeptides
25 and the phaseolin 3' region can be isolated as a restriction fragment. This fragment can then
10 be inserted into a unique restriction site of the vector carrying the marker gene.

20 To 50 µL of a 60 mg/mL 1 µm gold particle suspension is added (in order): 5 µL
25 DNA (1 µg/µL), 20 µL spermidine (0.1 M), and 50 µL CaCl₂ (2.5 M). The particie
30 preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the
supernatant removed. The DNA-coated particles are then washed once in 400 µL 70%
35 ethanol and resuspended in 40 µL of anhydrous ethanol. The DNA/particle suspension can
40 be sonicated three times for one second each. Five µL of the DNA-coated gold particles are
then loaded on each macro carrier disk.

45 Approximately 300-400 mg of a two-week-old suspension culture is placed in an
empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette.

50 20 For each transformation experiment, approximately 5-10 plates of tissue are normally
35 bombarded. Membrane rupture pressure is set at 1100 psi and the chamber is evacuated to a
40 vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the
45 retaining screen and bombardcd three times. Following bombardment, the tissue can be
divided in half and placed back into liquid and cultured as described above.

50 25 Five to seven days post bombardment, the liquid media may be exchanged with fresh
35 media, and eleven to twelve days post bombardment with fresh media containing 50 mg/mL
40 hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post
45 bombardment, green, transformed tissue may be observed growing from untransformed,
necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into
50 30 individual flasks to generate new, clonally propagated, transformed embryogenic suspension
cultures. Each new line may be treated as an independent transformation event. These
suspensions can then be subcultured and maintained as clusters of immature embryos or
regenerated into whole plants by maturation and germination of individual somatic embryos.

EXAMPLE 6

Expression of Chimeric Genes in Microbial Cells

55 35 The cDNAs encoding the instant polypeptides can be inserted into the T7 *E. coli*
expression vector pBT430. This vector is a derivative of pET-3a (Rosenberg et al. (1987)
50 40 *Gene* 56:125-135) which employs the bacteriophage T7 RNA polymerase/T7 promoter

5 system. Plasmid pBT430 was constructed by first destroying the EcoR I and Hind III sites in
pET-3a at their original positions. An oligonucleotide adaptor containing EcoR I and
Hind III sites was inserted at the BamHI site of pET-3a. This created pET-3aM with
additional unique cloning sites for insertion of genes into the expression vector. Then, the

10 5 Nde I site at the position of translation initiation was converted to an Nco I site using
oligonucleotide-directed mutagenesis. The DNA sequence of pET-3aM in this region,
5'-CATATGG, was converted to 5'-CCCATGG in pBT430.

15 Plasmid DNA containing a cDNA may be appropriately digested to release a nucleic
acid fragment encoding the protein. This fragment may then be purified on a 1% NuSieve

20 10 GTG™ low melting agarose gel (FMC). Buffer and agarose contain 10 µg/ml ethidium
bromide for visualization of the DNA fragment. The fragment can then be purified from the
agarose gel by digestion with GELase™ (Epicentre Technologies) according to the
manufacturer's instructions, ethanol precipitated, dried and resuspended in 20 µL of water.

25 20 Appropriate oligonucleotide adapters may be ligated to the fragment using T4 DNA ligase

30 15 (New England Biolabs, Beverly, MA). The fragment containing the ligated adapters can be
purified from the excess adapters using low melting agarose as described above. The vector
pBT430 is digested, dephosphorylated with alkaline phosphatase (NEB) and deproteinized
25 with phenol/chloroform as described above. The prepared vector pBT430 and fragment can
then be ligated at 16°C for 15 hours followed by transformation into DH5 electrocompetent
30 cells (GIBCO BRL). Transformants can be selected on agar plates containing LB media and
100 µg/mL ampicillin. Transformants containing the gene encoding the instant polypeptides
35 are then screened for the correct orientation with respect to the T7 promoter by restriction
enzyme analysis.

40 For high level expression, a plasmid clone with the cDNA insert in the correct
45 25 orientation relative to the T7 promoter can be transformed into *E. coli* strain BL21(DE3)
(Studier et al. (1986) *J. Mol. Biol.* 189:113-130). Cultures are grown in LB medium
35 containing ampicillin (100 mg/L) at 25°C. At an optical density at 600 nm of approximately
1, IPTG (isopropylthio-β-galactoside, the inducer) can be added to a final concentration of
40 0.4 mM and incubation can be continued for 3 h at 25°. Cells are then harvested by
centrifugation and re-suspended in 50 µL of 50 mM Tris-HCl at pH 8.0 containing 0.1 mM
45 35 DTT and 0.2 mM phenyl methylsulfonyl fluoride. A small amount of 1 mm glass beads can
be added and the mixture sonicated 3 times for about 5 seconds each time with a microprobe
sonicator. The mixture is centrifuged and the protein concentration of the supernatant
determined. One µg of protein from the soluble fraction of the culture can be separated by
35 SDS-polyacrylamide gel electrophoresis. Gels can be observed for protein bands migrating
at the expected molecular weight.

Claims

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CLAIMS

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What is claimed is:

1. An isolated polynucleotide comprising a nucleotide sequence encoding a first polypeptide of at least 100 amino acids that has at least 80% identity based on the Clustal 5 method of alignment when compared to a polypeptide selected from the group consisting of a *Momordica charantia* thioredoxin polypeptide of SEQ ID NO:2, a *Catalpa speciosa* thioredoxin polypeptide of SEQ ID NO:4, a soybean thioredoxin polypeptide of SEQ ID NO:6, a soybean thioredoxin polypeptide of SEQ ID NO:8 and a *Vernonia* thioredoxin polypeptide of SEQ ID NO:10.
- 10 2. An isolated polynucleotide comprising the complement of polynucleotide of Claim 1.
3. The isolated polynucleotide of Claim 1, wherein the nucleotide sequence comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, 3, 5, 7 and 9 that codes for the polypeptide selected from the group consisting of SEQ ID NO:2, 15 4, 6, 8 and 10.
4. The isolated polynucleotide of Claim 1 which is DNA.
5. The isolated polynucleotide of Claim 1 which is RNA.
- 20 6. A chimeric gene comprising the isolated polynucleotide of Claim 1 or Claim 2 operably linked to suitable regulatory sequences.
7. An isolated host cell comprising the chimeric gene of Claim 6.
8. An isolated host cell comprising an isolated polynucleotide of Claim 1.
9. The isolated host cell of Claim 8, wherein the host cell is yeast.
10. The isolated host cell of Claim 8, wherein the host cell is a bacterial cell.
11. The isolated host cell of Claim 8, wherein the host cell is a plant cell.
- 25 12. A virus comprising the isolated polynucleotide of Claim 1.
13. A process for producing an isolated host cell comprising the chimeric gene of claim 6, the process comprising either transforming or transfecting an isolated compatible host cell with the chimeric gene of Claim 6.
14. A thioredoxin polypeptide of at least 100 amino acids comprising at least 80% 30 homology based on the Clustal method of alignment compared to a polypeptide selected from the group consisting of SEQ ID NO:2, 4, 6, 8 and 10.
15. A method of selecting an isolated polynucleotide that affects the level of expression of a thioredoxin polypeptide in a plant cell, the method comprising the steps of: 40 constructing an isolated polynucleotide comprising a nucleotide sequence of at least one of 30 contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NO:1, 3, 5, 7, 9 and the complement of such nucleotide sequences; 45 introducing the isolated polynucleotide into a plant cell.

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5 measuring the level of thioredoxin polypeptide in the plant cell containing the polynucleotide; and

10 comparing the level of thioredoxin polypeptide in the plant cell containing the isolated polynucleotide with the level of thioredoxin polypeptide in a plant cell that does not 5 contain the polynucleotide.

15 16. The method of Claim 15 wherein the isolated polynucleotide comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:1, 3, 5, 7 and 9 that codes for the polypeptide selected from the group consisting of SEQ ID NO:2, 4, 6, 8 and 10.

17. The method of Claim 15 wherein the isolated polynucleotide is DNA.

18. The method of Claim 15 wherein the isolated polynucleotide is RNA.

20 19. The method of Claim 15 wherein the isolated polynucleotide is a chimeric genc comprising the nucleotide sequence operably linked to suitable regulatory sequences.

25 20. A method of selecting an isolated polynucleotide that affects the level of expression of thioredoxin polypeptide in a plant cell, the method comprising the steps of:

constructing the isolated polynucleotide of claim 1;

introducing the isolated polynucleotide into a plant cell;

20 25 measuring the level of thioredoxin polypeptide in the plant cell containing the polynucleotide; and

25 30 comparing the level of thioredoxin polypeptide in the plant cell containing the isolated polynucleotide with the level of thioredoxin polypeptide in a plant cell that does not contain the polynucleotide.

35 30 21. A method of obtaining a nucleic acid fragment encoding a substantial portion of a thioredoxin gene comprising the steps of:

35 35 25 synthesizing an oligonucleotide primer comprising a nucleotide sequence of at least one of 40 contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NO:1, 3, 5, 7, 9 and the complement of such nucleotide sequences; and

amplifying a nucleic acid sequence using the oligonucleotide primer.

40 30 35 22. A method of obtaining a nucleic acid fragment encoding all or a substantial portion of the amino acid sequence encoding a thioredoxin protein comprising the steps of:

45 35 35 probing a cDNA or genomic library with an isolated polynucleotide comprising a nucleotide sequence of at least one of 30 contiguous nucleotides derived from a nucleotide sequence selected from the group consisting of SEQ ID NO:1, 3, 5, 7, 9 and the complement of such nucleotide sequences,

50 45 35 identifying a DNA clone that hybridizes with the isolated polynucleotide; isolating the identified DNA clone; and

5 sequencing the cDNA or genomic fragment that comprises the isolated DNA
clone.

10 23. The isolated polynucleotide of Claim 1, wherein the first polypeptide is
compared to the *Momordica charantia* thioredoxin polypeptide of SEQ ID NO:2.

15 24. The isolated polynucleotide of Claim 1, wherein the first polypeptide is
compared to the *Catákva speciosa* thioredoxin polypeptide of SEQ ID NO:4.

20 25. The isolated polynucleotide of Claim 1, wherein the first polypeptide is
compared to the soybean thioredoxin polypeptide of SEQ ID NO:6.

25 26. The isolated polynucleotide of Claim 1, wherein the first polypeptide is
compared to the soybean thioredoxin polypeptide of SEQ ID NO:8.

30 27. The isolated polynucleotide of Claim 1, wherein the first polypeptide is
compared to the *Vernonia* thioredoxin polypeptide of SEQ ID NO:10.

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SEQUENCE LISTING

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 Thorpe, Cathy

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